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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Toshio Sone
Serial No. : 09/308,027
Filed : August 16, 1999
Title : PEPTIDE-BASED IMMUNOTHERAPEUTIC AGENT

Art Unit : 1644
Examiner : P. Huynh

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RESPONSE TO NOTICE OF INCOMPLETE RESPONSE

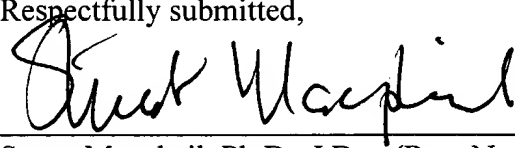
This submission is in response to the Office Action mailed March 2, 2005 (copy enclosed), regarding an Information Disclosure Statement (IDS) filed after the issue fee was paid (on January 27, 2005). The Office Action and Examiner Huynh (in a telephone conversation with Applicant's undersigned representative on March 14, 2005) indicated that, despite acknowledgement of its receipt by the U.S. Patent and Trademark Office (USPTO) by return postcard, she did not receive a copy of the reference listed in a PTO Form 1449 submitted with the IDS. Applicant hereby resubmits copies of the IDS, the PTO Form 1449, and the reference listed in the PTO Form 1449. Also enclosed is a copy of the above-mentioned return postcard stamped received by the USPTO on January 31, 2005.

Please accept these papers as part of the record in this patent application.

Respectfully submitted,

Date: 3/16/05

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Stuart Macphail, Ph.D., J.D. (Reg. No. 44,217)

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/308,027	08/16/1999	TOSHIO SONE	14883-031001-MI-808PCT-US	5615

7590

03/02/2005

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EXAMINER

HUYNH, PHUONG N

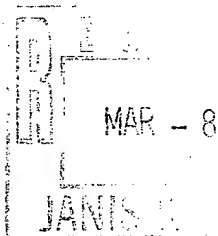
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PAPER NUMBER

1644

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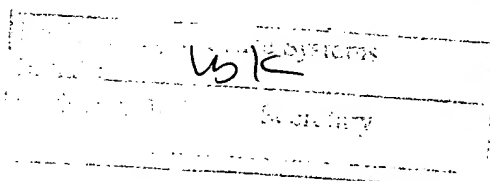
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APPLICATION NO./ CONTROL NO.	FILING DATE	FIRST NAMED INVENTOR / PATENT IN REEXAMINATION	ATTORNEY DOCKET NO.
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Applicant's information disclosure statement of 1/31/05 was filed after the issue fee was paid. Information disclosure statements filed after payment of the issue fee will not be considered, but will be placed in the file. However, the application may be withdrawn from issue in order to file a request for continued examination (RCE) under 37 CFR 1.114 upon the grant of a petition under 37 CFR 1.313(c)(2), or a continuing application under 37 CFR 1.53(b) (or a continued prosecution application (CPA) under 37 CFR 1.53(d) if the CPA is for a design patent and the prior application of the CPA is a design application) upon the grant of a petition filed under the provisions of 37 CFR 1.313(c)(3). Alternatively, the other provisions of 37 CFR 1.313 may apply, e.g., a petition to withdraw the application from issue under the provisions of 37 CFR 1.313(c)(1) may be filed together with an unequivocal statement by the applicant that one or more claims are unpatentable over the information contained in the statement. The information disclosure statement would then be considered upon withdrawal of the application from issue under 37 CFR 1.313(c)(1).

Further, the information disclosure statement filed 1/31/05 fails to comply with 37 CFR 1.98(a)(2), which requires a legible copy of each U.S. and foreign patent; each publication or that portion which caused it to be listed; for each cited pending U.S. application, the application specification including the claims, and any drawing of the application, or that portion of the application which caused it to be listed including any claims directed to that portion and all other information or that portion which caused it to be listed. It has been placed in the application file, but the information referred to therein has not been considered.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Phuong Huynh "NEON" whose telephone number is (571) 272-0846. The examiner can normally be reached Monday through Friday from 9:00 am to 5:30 p.m. A message may be left on the examiner's voice mail service. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Christina Chan can be reached on (571) 272-0841. The IFW official Fax number is (571) 273-8300.

Any information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Phuong N. Huynh, Ph.D.
Patent Examiner
February 24, 2005

Christina Chan
CHRISTINA CHAN
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600

Substitute Form PTO-1449
(modified)U.S. Department of Commerce
Patent and Trademark OfficeAttorney's Docket No.
14883-031001Application No.
09/308,027**Information Disclosure Statement
by Applicant**

(Use several sheets if necessary)

(37 CFR §1.98(b))

Applicant
Toshio Sone et al.Filing Date
August 16, 1999Group Art Unit
1644**U.S. Patent Documents**

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	AA						
	AB						
	AC						
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Foreign Patent Documents or Published Foreign Patent Applications

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	AL							
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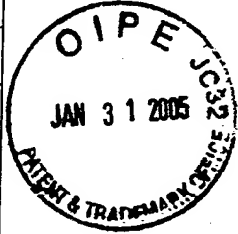
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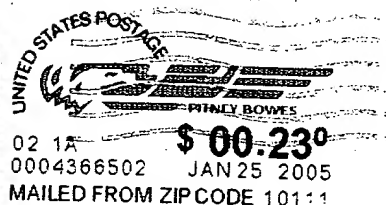
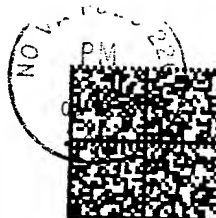
Examiner Initial	Desig. ID	Document
	AQ	Hino et al. "Immunodominance of seven regions of a major allergen, Cry j 2, of Japanese cedar pollen for T-cell immunity" Allergy, 51(9):621-632 (1996)
	AR	
	AS	
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Date Considered

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Attorney's Docket No. 14883-031001	Express Mail Label No.	Mailing Date January 27, 2005	For PTO Use Only <i>Do Not Mark in This Area</i> 
Application No. 09/308,027	Filing Date August 16, 1999	Attorney/Secretary Init SXM/myw	
Title of the Invention PEPTIDE-BASED IMMUNOTHERAPEUTIC AGENT			
Applicant Toshio Sone et al.			
Client Reference No. M1-808PCT-US			
Enclosures · Information Disclosure Statement (1 page) · Form PTO-1449 (1 page) · Documents listed on the Form PTO-1449 (1 document)			



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INFORMATION DISCLOSURE STATEMENT

Applicants submit the reference listed on the attached form PTO-1449. The reference was cited in the Korean application corresponding to the present application in an Office Action mailed December 21, 2004.

This statement is filed under 37 C.F.R. § 1.97(i). Please apply any charges or credits to Deposit Account No. 06-1050.

Respectfully submitted,

Date: _____

1/27/05

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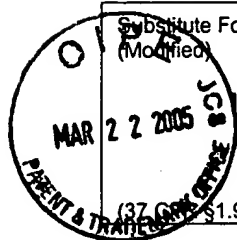
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 U.S. Department of Commerce
 Patent and Trademark Office

 Attorney's Docket No.
 14883-031001

 Application No.
 09/308,027

**Information Disclosure Statement
 by Applicant**

(Use several sheets if necessary)

 Applicant
 Toshio Sone et al.

 Filing Date
 August 16, 1999

 Group Art Unit
 1644

(37 CFR 1.98(b))

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	AA						
	AB						
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Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation	
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	AL							
	AM							
	AN							
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	AP							

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document
	AQ	Hino et al., "Immunodominance of seven regions of a major allergen, Cry j 2, of Japanese cedar pollen for T-cell immunity" Allergy, 51(9):621-632 (1996)
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	AT	

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Date Considered

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Immunodominance of seven regions of a major allergen, Cry j 2, of Japanese cedar pollen for T-cell immunity

Hashiguchi S, Hino K, Taniguchi Y, Kurimoto M, Fukuda K, Ohyama M, Fujiyoshi T, Sonoda S, Nishimura Y, Yamada G, Sugimura K. Immunodominance of seven regions of a major allergen, Cry j 2, of Japanese cedar pollen for T-cell immunity. *Allergy* 1996; 51: 621-632. © Munksgaard 1996.

The immunodominant regions of the Japanese cedar pollen allergen Cry j 2 for T-cell immunity were determined with whole peripheral blood lymphocytes (PBL) derived from seven allergic patients and three nonallergic subjects. Cry j 2-stimulated T-cell proliferation was inhibited by anti-HLA-DR, but not by anti-HLA-DQ antibody, indicating that the responding T cells recognized the allergen peptides associated with HLA-DR molecules. It was found that seven regions of Cry j 2, i.e., regions corresponding to amino acid numbers 1-26, 70-84, 151-167, 187-203, 252-279, 283-314, and 345-362, were immunodominant for T-cell proliferation. Thus, Cry j 2 bears a limited number of immunodominant regions despite polymorphic features of HLA-DR in the immune system. This suggests the possibility of molecularly designing Cry j 2 antagonists that could downregulate allergic reactions to Japanese cedar pollen.

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Key words: allergen; immunodominance; Japanese cedar pollen; pollinosis; T-cell epitope.

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Accepted for publication 28 March 1996

Allergic responses to Japanese cedar (*Cryptomeria japonica*) pollen are to a very large extent directed toward two components, the Cry j 1 and Cry j 2 allergens, as measured by IgE reactions. These two major allergens have been isolated (1, 2), and the primary amino acid sequences have recently been clarified by molecular cloning (3, 4).

Recent studies have demonstrated that partial alteration of the amino acid sequence of T-cell epitopes induced a stimulatory signal that was qualitatively different (5-8). It is conceivable that immunotherapy using peptides representing dominant T-cell epitopes could modulate the T-cell response of allergic patients and therefore prevent the production of IgE antibodies at the earliest stage.

In this study, we determined the T-cell epitopes of Cry j 2 by using enzyme-digested Cry j 2 fragments, and then chemically synthesizing the

oligopeptides. Peripheral blood lymphocytes (PBL) derived from patients with pollinosis and from healthy donors were used in this system to investigate the response of bulk populations of CD4⁺ T cells in comparison with that of a selected number of antigen-specific T-cell clones. This could provide important information for developing immunotherapy using allergen peptides.

Here we demonstrate first that both allergic and nonallergic subjects display T-cell reactivity toward a limited number of T-cell epitopes of the allergy protein Cry j 2, and, second, that seven regions are prominent in the immunologic potency of Cry j 2 in spite of HLA-DR polymorphism. This may be attributed to the combined effects of amino acids in immunodominant epitopes which are responsible for binding to HLA class II molecules and T-cell receptors in the bulk population of T cells in the periphery (7, 8).

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Thus, a limited number of immunodominant Cry j 2 epitopes may lead to a molecular design of the Cry j 2 antagonist that could downregulate the allergic reaction to Japanese cedar pollen.

Material and methods

Patients and controls

Nine pollinosis patients and three nonallergic donors were studied during the allergy season (February–May). These donors were selected on the basis of clinical examination, routine skin prick tests, nasal provocation tests, and eosinophil staining of nasal smears. HLA class I (A, B, C) and class II (DR) antigens were typed by the microcytotoxicity test using defined anti-HLA antisera, as previously described (9). For the terminology of the HLA-DR, DO alleles were used according to the WHO Nomenclature Committee (10). HLA class II alleles were genotyped by the SMITEST system (Sumitomo Metal Industries, Tokyo, Japan) based on the polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) method (11, 12). Briefly, high-molecular-weight genomic DNAs from PBL were prepared by proteinase K digestion and phenol/chloroform extraction, as previously described (13). The second exons of the DRB1 and DQB1 genes were selectively amplified by PCR with group-specific primers for 30–35 cycles consisting of 1 min at 94°C, 1 min at 55–62°C, and 1 min at 72°C. A volume of 10 µl of the PCR products was digested with a set of allele-specific restriction endonucleases and was subjected to electrophoresis in 10% polyacrylamide gels to determine the RFLP patterns of the HLA class II alleles. The results of the HLA typing are shown in Table 1.

Purification of Cry j 2 and enzyme-digested Cry j 2

Cry j 2 protein was isolated from a Japanese cedar pollen extract by affinity chromatography using a monoclonal antibody specific for Cry j 2, mAb N26. Partial digestion with V8 protease (from *Staphylococcus*; Sigma, St Louis, MO, USA) or lysylendo-peptidase (from *Acromobacter*; Seikagaku Co., Tokyo, Japan), and high-performance liquid chromatography (HPLC) purification of peptide fragments were performed (Fig. 1), as previously described (3). The amino acid sequence was determined on a protein sequencer (model 473A, Applied Biosystems, Foster City, CA, USA). Regarding the peptide fraction, the purity of the amino acid sequence was estimated at about 90% unless otherwise described. In the amino acid sequence described by Namba et al. (3), no. 51 is

Table 1. HLA donor types

		Serology	DNA typing	
Subjects		DR	DRB1*	DQB1*
Allergic	T. I.	6/9	0901/1403	0301/03032
	F. N.	4/8	0405/0803	0401/0801
	E. Y.	4/6	0403/1302	0401/0604
	M. H.	4/-	0405/-	0401/-
	T. H.	ND	0101/1302	0501/0604
	T. M.	4/6	0405/1405	0401/0531
Nonallergic	J. T.	ND	0101/1405	0301/0301
	S. H.	4/6	0406/1301	0303/0803
	H. A.	8/9	0803/0901	0303/0601
	N. T.	4/6	0406/1301	0302/0603

ND: not determined.

labeled serine. In this study, that serine has been designated as no. 1.

Peptide synthesis and characterization

A set of 94 overlapping 14-mer peptides spanning the entire length of Cry j 2 was synthesized on polyethylene rods (pins) by the multipin technique (Chiron Mimotopes Pty. Ltd, Victoria, Australia), as previously described (14). The peptides overlapped each other by 10 residues. Reverse-phase HPLC and amino acid composition analyses showed the purity of the preparation to be 60–70%. Several immunodominant peptides were synthesized with Peptide Synthesizer, 9050-plus (Millipore, Bedford, MA, USA) and purified by reverse-phase HPLC using a C18 column (19×150 mm, µ Bondasphere, 5 µ C18 100A, Waters chromatography, Marlboro, MA, USA) under a 10–80% gradient of acetonitrile in 0.1% trifluoroacetic acid in water, at a flow rate of 10 ml/min, as described by us previously (3, 15). The amino acid sequence was verified on a model 473A protein sequencer (Applied Biosystems, Foster City, CA, USA).

T-cell proliferation assay

To examine T-cell proliferation, PBL (6×10^4 /well) were cultured in triplicate in 96-well, round-bottomed plates (Corning, Inc, Corning, NY, USA) in 200 µl RPMI-1640 medium (ICN Biomedicals, Inc, Costa Mesa, CA, USA) supplemented with 7.5% heat-inactivated AB human serum (Biocell, Carson, CA, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin and 2-mercaptoethanol (4×10^{-5} M) and were stimulated with Cry j 1, Cry j 2 (0.1 µM), or PPD (0.3 µg/ml, Nippon BCG, Tokyo, Japan). Cell proliferation was monitored by the addition

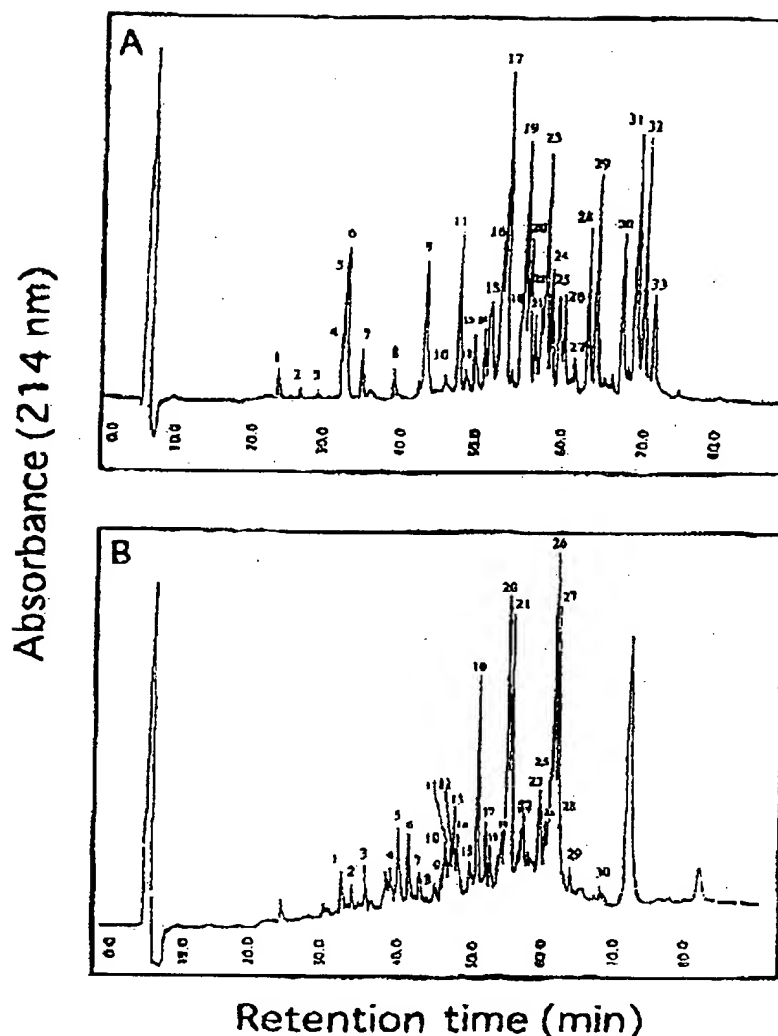


Fig. 1. Reverse-phase HPLC purification of enzyme-digested Cry j 2. A) Lysylendopeptidase-digested Cry j 2. B) V8 protease-digested Cry j 2.

of ^3H -thymidine (0.5 μCi per well, Amersham, St Louis, MO, USA) during the last 18 h of incubation, and its incorporation was measured on a liquid scintillation counter. Anti-HLA-DR (Hu-4; anti-HLA-DR framework mAb) and anti-HLA-DQ (mixture of three monoclonal antibodies: Hu-11, anti-HLA-DQ1 mAb; Hu-18, anti-HLA-DQ3 mAb; and Hu-46, anti-HLA-DQ4 mAb) antibodies (ascites) were used to examine the MHC restriction of the response (16).

Flow cytometry analysis

Cytofluorographic analysis was done on an EPICS XL (Coulter, Hialeah, FL, USA) using phycoerythrin-conjugated CD8 (Nippon Becton-Dickinson,

Tokyo, Japan) and fluorescein-conjugated CD4 antibodies (Coulter Immunology, Tokyo, Japan).

Enrichment of CD4^+ T cells

Briefly, PBL were resuspended in phosphate-buffered saline (PBS) containing 10% fetal calf serum (FCS) (10^7 cells per 80 μl of PBS/FCS), mixed with 10^7 cells/20 μl of anti-CD8 antibody Microbeads (Milenyi Biotec GmbH, Bergisch Gladbach, Germany) and were incubated on ice for 30 min. The labeled cells were washed once with PBS/FCS and resuspended in 0.5 ml PBS/FCS before application to a MACS column (Milenyi Biotec GmbH, Bergisch Gladbach, Germany). The CD8-negative fraction containing

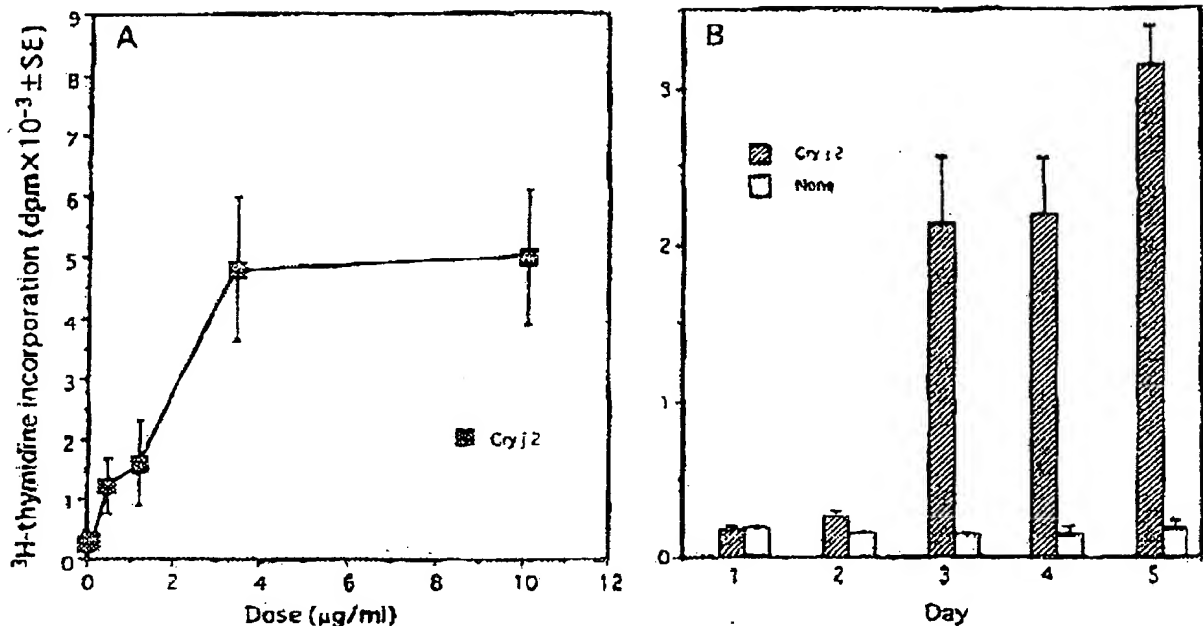


Fig. 2. T-cell proliferation in response to Japanese cedar pollen allergen Cry j 2. PBL were stimulated with varying doses of affinity-purified Cry j 2. T-cell proliferation was monitored each day by pulsing cells with ^3H -labeled thymidine, as described in Material and methods section.

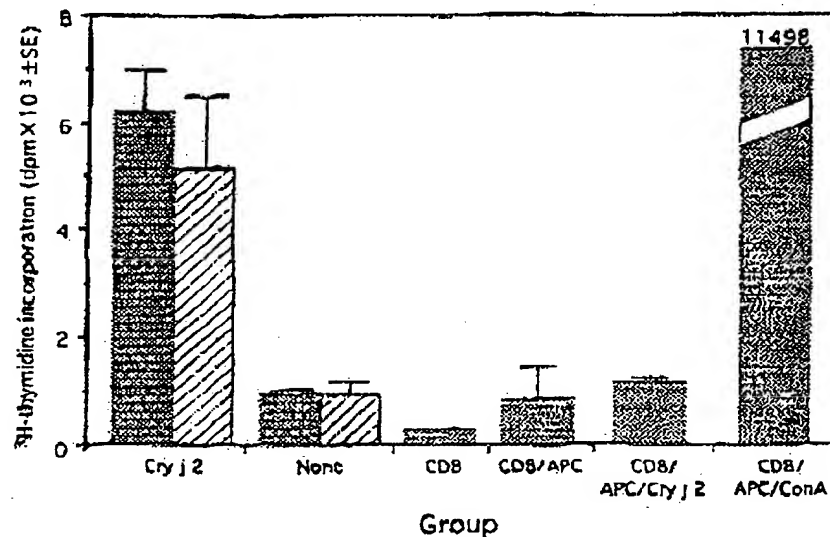


Fig. 3. CD4^+ T-cell response to Cry j 2. ■: 6×10^4 PBL, ▨: 6×10^4 CD4^+ cells, □: 2×10^4 CD8^+ cells in presence or absence of 10^5 Gy-irradiated 4×10^4 PBL. Cells were stimulated with Cry j 2 or concanavalin A (2.0 $\mu\text{g/ml}$). Number above solid bar indicates mean value (dpm) of triplicate cultures.

the non- CD8^+ T-cells was collected. This step was repeated twice to ensure complete CD8^+ T-cell depletion. Yields of the CD8^+ -depleted, CD4^+ -enriched cell population were consistently 45–55% of the starting PBL. This is referred to as the CD4^+ cell population which contains less than 2.0% CD8^+ cells.

Results

T-cell proliferation in response to Cry j 2

To characterize the T-cell response to Cry j 2, PBL were stimulated with varying doses of HPLC-purified Cry j 2, ranging from 0.003 μM (0.1 μg)

Cry j 2 allergen T-cell epitopes

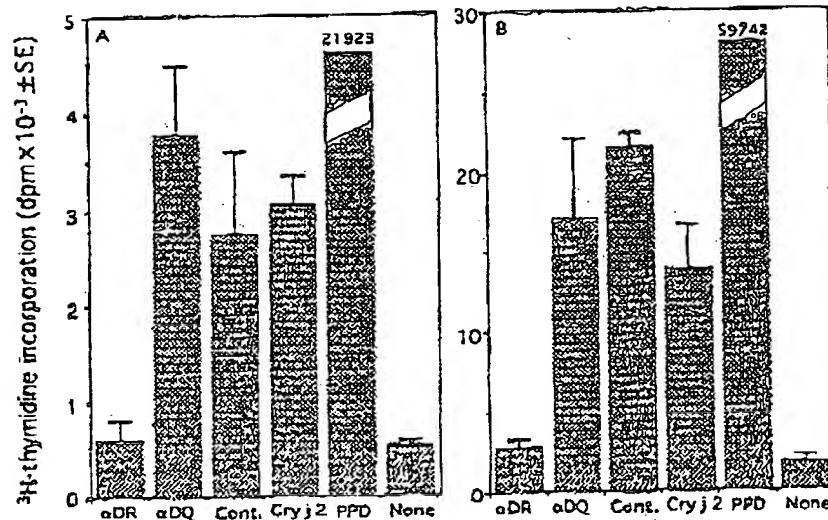


Fig. 4. HLA-DR restriction of Cry j 2-specific T-cell proliferation. A) PBL from allergic patient M. H. (DRB1*0405/-); B) PBL from allergic patient J. T. (DRB1*0101/1406). PBL were stimulated with 0.3 μ g/ml PPD, 0.1 μ M Cry j 2 in presence or absence of 1:200 diluted monoclonal antibodies, control normal mouse serum, anti-DQ (mixture of Hu-11, Hu-18, and Hu-46), and anti-DR (Hu-4) (Ref. 16). Vertical bars indicate standard error (SE) of triplicate cultures. Numbers above solid bars indicate mean values of disintegrations per minute (dpm) for triplicate cultures of responses.

to 0.3 μ M (10 μ g). As shown in Fig. 2, the optimal concentration of allergens was 0.1 μ M (3 μ g/ml) (Fig. 2a). The proliferative responses were measured by day 5 (Fig. 2b). Thereafter, the responses were assayed on day 5. To determine the lymphocyte subsets responding to Cry j 2, CD8⁺ cells were depleted from PBL by the magnetic separation method. CD8⁺ cell-depleted PBL contains 40% CD4⁺ cells and less than 2% CD8⁺ cells by flow-cytometry analysis. The CD8⁺ cell fraction contains 98% CD8⁺ cells and less than 2% CD4⁺ cells. We tested one nonallergic and two allergic donors. A representative result is presented in Fig. 3. CD4⁺ cells responded to Cry j 2 as well as PPD. In contrast, CD8⁺ cells showed no proliferative response even in the presence of antigen-presenting cells (APC). The APC supported the mitogenic response of CD8⁺ cells to ConA, indicating their ability of antigen presentation. The magnitude of the response of CD8⁺ cell-depleted cells was almost the same as that of whole PBL stimulated with Cry j 2. The response to Cry j 1 also showed the identical features (data not shown). When the HLA restriction was examined in the seven allergic patients, these T-cell proliferative responses were inhibited more than 90% by the addition of anti-HLA-DR antibody, but not by anti-HLA-DQ antibodies. Two representative patients, M. H. and J. T. in Fig. 4, indicated that the responses were restricted by HLA-DR molecules.

T-cell response to enzyme-digested Cry j 2 fragments

Cry j 2 was partially digested with lysylendopeptidase (L) or V8 protease (V), in which 33 or 30 fractions, respectively, were separated and tested for T-cell stimulatory activity (Fig. 1). We examined seven patients and two of the three nonallergic donors for anti-Cry j 2 T-cell response by stimulating each fraction at a concentration of 6.5 μ M. The amino acid sequences of all immunogenic fractions were also determined. It was noted that there were only a few fractions with prominent immunodominance and that the magnitude of responses was generally smaller than that of the intact Cry j 2 allergen (Fig. 5). We found five patients in whom one or two fractions showed prominent stimulatory effects (S. H., M. D., M. H., E. Y., and H. A. in Fig. 5). Table 2 summarizes these results. We tentatively defined a positive response as being at least threefold higher than a nonstimulated response. Potentially immunodominant fractions were further examined for their activities by dose-response analysis. As shown in Fig. 6, the optimal concentration ranged from 1.8 to 10 μ M which was 10–100 times higher than required for stimulation with intact Cry j 2 (0.1 μ M). With lysylendopeptidase-digested Cry j 2, positive responses were obtained in L1, L23, and L25. Responses were hardly detected in any fractions in the other patients tested. In the cases of V8 protease-digested Cry j 2,

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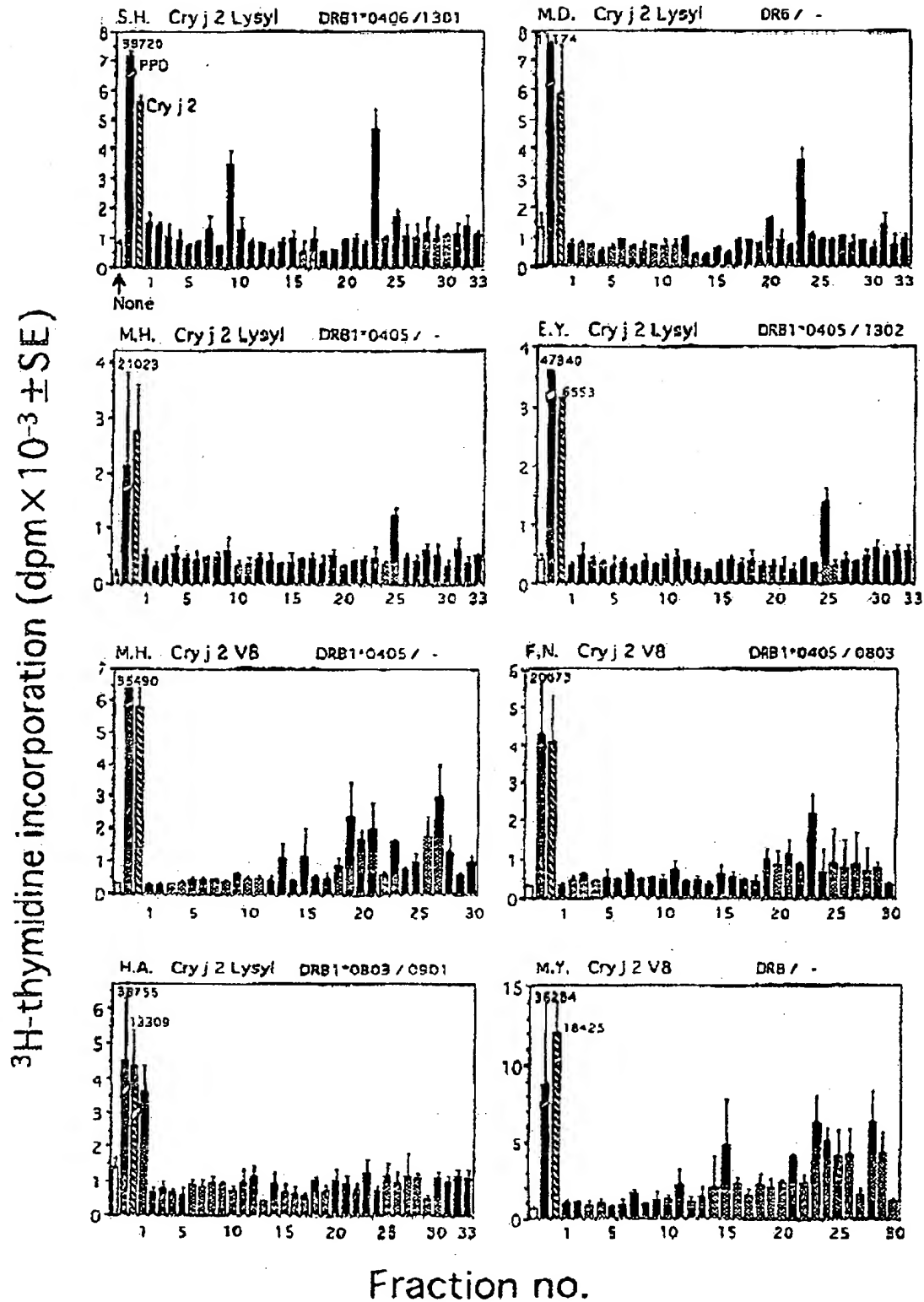


Fig. 5. Immunodominant fragments of enzyme-digested Cry j 2 in allergic and nonallergic subjects. S. H. and H. A. are nonallergic donors without allergic symptoms. M. D., M. H., E. Y., F. N., and M. Y. are allergic patients. Lysyl: lysylendopeptidase-digested Cry j 2; V8: V8 protease-digested Cry j 2. Vertical bars indicate standard error (SE) of triplicate cultures. Numbers above solid bars indicate mean values of dpm of responses.

Cry j 2 allergen T-cell epitopes

Table 2. T-cell proliferation in response to HPLC-purified enzyme-digested Cry j 2 fragments

Donor	Experiment	HLA-DR		Stimulants ¹		Sequence ²	% Response ³	SI ⁴		
		Serology	DNA typing		(μ M)					
Allergic M. M.	1	DR4/-	0405/-	L25	5.5	151-167	41	6.8		
				Cry j 2	0.1		100	15.2		
				PPD			(21 023 \pm 1720) ³	115.5		
				None			0 (182 \pm 58)	1.0		
	2			V19	5.5	ND	38	7.8		
				V20	5.5	268-279	25	5.5		
				V21	5.5	283-293	31	6.5		
				V23	5.5	5-9, 1-12 ⁵	24	5.2		
				V26	5.5	283-314	26	5.7		
				V27	5.5	348-383	49	6.8		
				Cry j 2	0.1		100	8.9		
				PPD			(35 489 \pm 9836)	118.4		
				None			0 (305 \pm 22)	1.0		
F. N.	1	DR4/DR8	0405/0803	V23	5.5	5-9, 1-12	48	5.9		
				Cry j 2	0.1		100	11.6		
				PPD			(20 537 \pm 1444)	59.5		
				None			0 (347 \pm 49)	1.0		
M. D.	1	DR6/-	ND	L23	5.5	70-84	51	2.8		
				Cry j 2	0.1		100	4.8		
				PPD			(11 174 \pm 760)	8.5		
				None			0 (1260 \pm 518)	1.0		
M. Y.	1	DR8/-	ND	V15	5.5	252-264	23	7.9		
				V21	5.5	283-293	19	5.7		
				V23	5.5	5-8, 1-12	32	10.4		
				V24	5.5	4-12	25	8.3		
				V25	5.5	ND	19	6.5		
				V26	5.5	283-314	20	6.8		
	2			V28	5.5	ND	32	10.4		
				V29	5.5	ND	20	7.0		
				Cry j 2	0.1		100	30.8		
				PPD			(38 284 \pm 5154)	60.2		
				None			0 (603 \pm 109)	1.0		
E. Y.	1	DR4/DR8	0405/1302	L25	5.5	151-167	17	3.4		
				Cry j 2	0.1		100	14.1		
				PPD			(47 340 \pm 5154)	101.8		
				None			0 (465 \pm 47)	1.0		
Nonallergic R. A.	1	DR8/DR8	0901/0803	L1	5.5	352-358	23	5.2		
				Cry j 2	0.1		100	19.4		
				PPD			(38 754 \pm 1795)	58.4		
				None			0 (687 \pm 148)	1.0		
	2					V15	1.8	252-284	86	3.0
						Cry j 2	0.1		100	3.4
						PPD			(37 858 \pm 4857)	82.1
						None			0 (461 \pm 185)	1.0
	3					V16	1.8	348-353	87	3.4
						Cry j 2	0.1		100	3.7
						PPD			(39 719 \pm 5013)	21.7
						None			0 (1282 \pm 268)	1.0
S. N.	1	DR4/DR6	0405/1301	L23	5.5	70-84	81	5.8		
				Cry j 2	0.1		100	6.9		
				PPD			(39 719 \pm 1830)	49.2		

Table cont. next page.

Table 2 cont. T-cell proliferation in response to HPLC-purified enzyme-digested Cry j 2 fragments

Donor	Experiment	HLA-DR		Stimulants ¹		Sequence ²	% Response ³	SI ⁴
		Serology	DNA typing		(μ M)			
				None			0 (607 \pm 109)	1.0
	2			L9	6.5	193-198	42	3.3
				Cry j 2	0.1		100	5.5
				PPD			(51357 \pm 5685)	53.6
				None			0 (955 \pm 55)	1.0

ND: not determined.

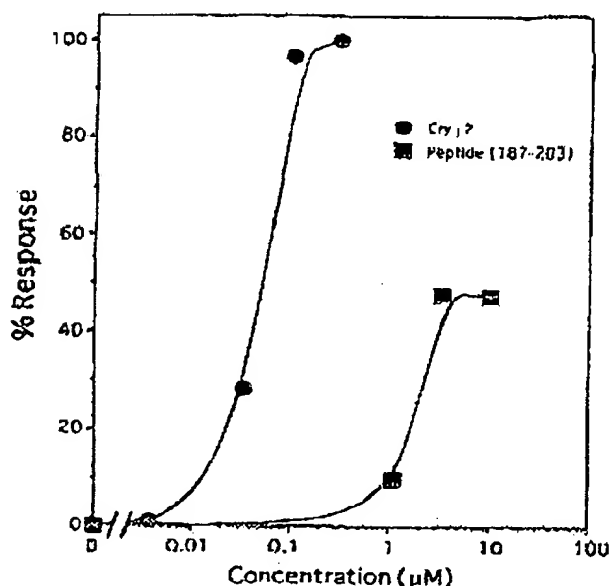
¹ Lysylendopeptidase- and VB protease-digested fragments are abbreviated as L and V, respectively. Cry j 2 indicates intact Cry j 2. PPD was used at 0.3 μ g/ml.² Position number of Cry j 2 amino acid sequence (3).³ Percent response = (mean value of dpm of experimental group - mean value of dpm of nonstimulated group)/mean value of dpm of intact Cry j 2 - stimulated group - mean value of dpm of nonstimulated group \times 100.⁴ SI = mean value of dpm of experimental group/mean value of dpm of nonstimulated group. Responses were confirmed independently by dose-response experiments.⁵ Number in parentheses indicates dpm \pm SD of triplicate experiments.⁶ Mixture of two fragments.

Fig. 6. Dose response of intact Cry j 2 and Cry j 2 peptide. Response to synthetic peptide (187-203) is shown. PBL were derived from patient S. H. PPD-induced response was 47484 ± 1902 dpm.

the response patterns were relatively more complex than those with lysylendopeptidase. V23 (1-12/5-9) was positive in allergic donors F. N. (DRB1*0405/0803) and M. Y. (DR8/-). L23 (70-84) was positive in allergic patients J. T. (DRB1*0101/1406) and M. D. (DR6/-) and in the nonallergic donor S. H. (DRB1*0406/1301). L25 (151-167) was positive in allergic patients M. H. (DRB1*0405/-) and E. Y. (DRB1*0405/1302), but negative in allergic patient F. N. (DRB1*0405/0803). L9 (193-198)

was positive in nonallergic donor S. H. (DRB1*0406/1301). V15 (252-264) was positive in allergic donor M. Y. (DR8/-). V20 (265-279) was positive in allergic donor, M. H. (0405/-). V21 (283-293) was positive in allergic donors M. H. (DRB1*0405/-) and M. Y. (DR8/-), who also showed responses to V26 (283-314). V27 (348-383) was positive in allergic patient M. H. (DRB1*0405/-). L1 (352-356) and V18 (348-353) were positive in nonallergic donor H. A. (DRB1*0803/0901), but negative in the other allergic patients tested. Fig. 7 shows the dose responses induced with the 1-12/5-9, 151-168, 252-279, 283-314, or 348-356 peptides. The amino acid sequences of V19, V25, V28, and V29 could not be determined due to their impurity. Both S. H. and N. T. were DRB1*0406/1301 and nonallergic donors, but S. H. showed a response to three fractions while N. T. showed no T-cell proliferative response even to intact Cry j 2 (data not shown).

Thus, there does not appear to be any correlation between the immunodominant fraction and the particular MHC class II type. Furthermore, there were cases in which both allergic and nonallergic subjects responded to the same fraction, suggesting the nonexistence of particular disclated fragments. There may, however, be a few disease-unrelated fragments such as L1 (352-356), L9 (193-198), and V18 (348-353). These results show that active fractions vary in each individual. However, relatively restricted fractions exhibited T-cell stimulatory activities.

T-cell response to synthetic Cry j 2 polypeptide

In parallel with the experiments using enzyme-digested Cry j 2, PBL was stimulated with a set

Cry j 2 allergen T-cell epitopes

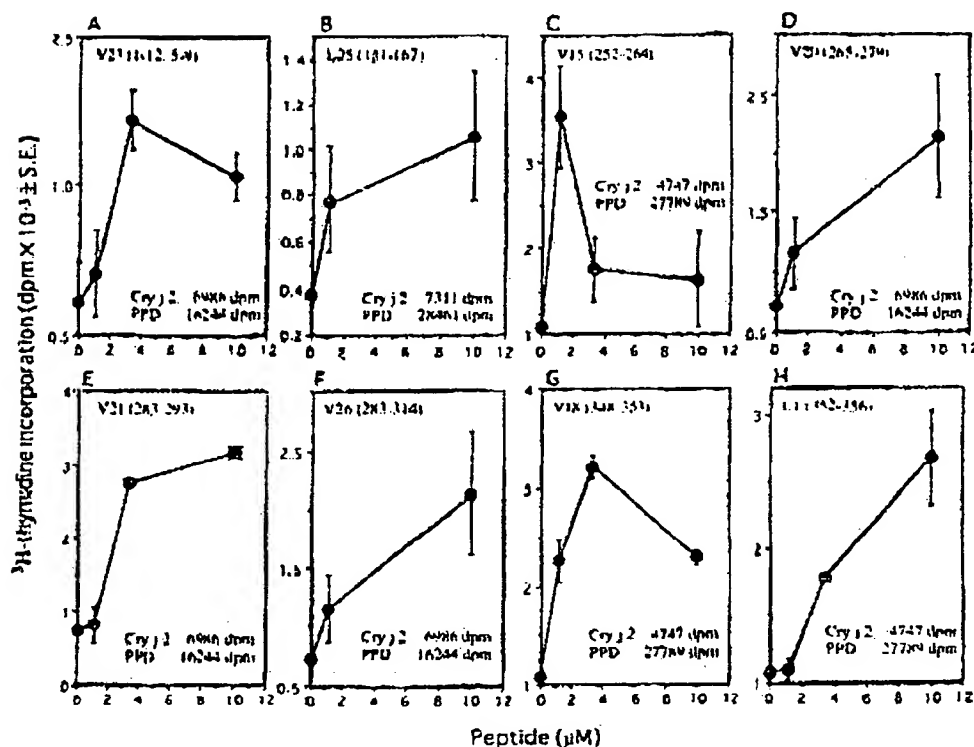


Fig. 7. Dose response of T-cell proliferation induced with enzyme-digested peptide fragments. A, D, E, F) PBL was derived from M.; B, C) PBL was derived from L.; G, H) PBL was derived from H. A.

of multipin synthesized 14-mer oligopeptides covering the entire Cry j 2 sequence (Fig. 8). A positive fraction was tentatively selected when the neighboring fractions induced proliferation, although a single positive fraction could have been excluded. Allergic patient J. T. exhibited a prominent immuno-dominance in fraction 73-86. Several other fractions showed weaker responses in the regions of 129-186 and 245-262. Nonallergic donor S. H. responded to three fractions, 13-26, 73-86, and 193-206. The stimulatory activity of the 345-358 or 349-362 multipin peptides is shown in Fig. 9a. Since fractions prepared by the multipin method were about 60% pure by HPLC, these results were confirmed by dose-response experiments using reversed phase-HPLC-purified polypeptide preparations synthesized on a Millipore peptide synthesizer (over 98% purity). The proliferative responses induced with the 187-203 or 69-85 peptides are shown in Fig. 9b. These results were consistent with those of enzyme-digested Cry j 2 fragments (Table 2 and Fig. 9). Table 3 shows the immunodominant regions of Cry j 2 and the amino acid sequence, respectively.

Discussion

To investigate the response pattern of bulk populations of CD4^+ T cells in comparison with a selected number of allergen-specific T-cell clones to the major allergen of Japanese cedar pollen (Cry j 2), PBL from allergic or nonallergic subjects were stimulated with a series of Cry j 2 peptide fragments to measure T-cell proliferation *in vitro*. Not only pollinosis patients but also two nonallergic subjects with no pollinosis symptoms showed remarkable T-cell proliferation in response to Cry j 2. All subjects were tested for Cry j 1- and Cry j 2-specific IgE antibody titers in serum by ELISA (17). These antibodies were not detected in non-allergic subjects (data not shown). The responding T cells were CD4^+ , but not CD8^+ (Fig. 3), suggesting that T cells activated by Cry j 1 or Cry j 2 in either allergic or nonallergic subjects might be distinct and dose not simply correlated with allergic status.

Seven allergic patients and one of the three nonallergic donors were tested for MHC restriction. The anti-Cry j 2 T-cell responses in these cases were inhibited by the addition of anti-

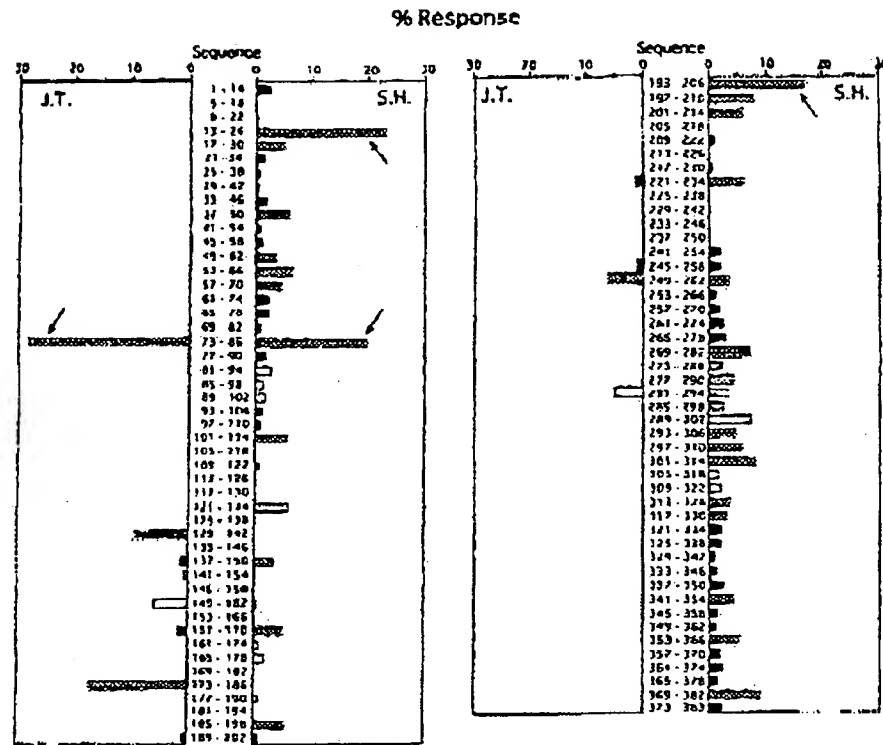


Fig. 8. Immunodominant Cry j 2 peptides (14 mer) synthesized by multipin method. Cry j 2 sequence number: see Ref. 3. PBL were stimulated with 1-2.5 μ M (\square), 2.5-5 μ M (\blacksquare), 5-7.5 μ M (\boxplus), or 7.5-10 μ M (\boxminus) of each peptide because yield of each fraction varied after synthesis by multipin method. Percent response: see Table 2 footnotes. Arrows indicate positive responses confirmed by dose-response experiments. Cry j 2-induced or nonstimulated response, 4726 ± 1295 or 1300 ± 14 dpm in J. T. and 17731 ± 2140 or 715 ± 301 dpm in S. H.

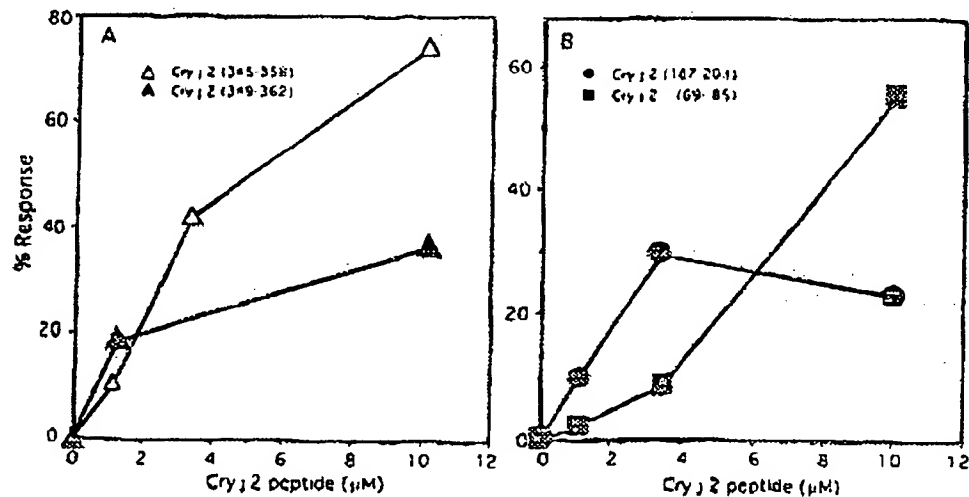


Fig. 9. Dose-response experiment of chemically synthesized Cry j 2 peptides. Percent response: see Table 2 footnote. Number in parentheses indicates amino acid sequence numbers of Cry j 2. A) Peptides prepared by multipin method (Δ) and (\blacktriangle), PBL from H. A.; nonstimulated response: 1282 dpm, Cry j 2-stimulated response: 4747 dpm. B) Peptides prepared by peptide synthesizer (\blacksquare), PBL from M. D.; nonstimulated response: 800 dpm, Cry j 2-stimulated response: 5825 dpm (\bullet). PBL from S. H.; nonstimulated response: 1750 dpm, Cry j 2-stimulated response: 17546 dpm.

Cry j 2 allergen T-cell epitopes

Table 3. Immunodominant regions of Cry j 2 for T-cell response

Regions	Cry j 2 sequences	Positive sequence (serology or DNA typing)		
		Enzyme digests	Multipin	Peptide synthesizer
1	1-26	1-12, 5-9 (0405/0803) 1-12, 5-9 (0405/-) 1-12, 5-9 (DRB/-) 13-16 (0406/1301)	13-26 (0406/1301)	13-26 (0406/1301)
2	70-84	70-84 (0406/1301)	73-86 (0406/1301) 73-86 (0101/1406)	69-85 (0406/1301) 69-85 (0101/1406)
3	151-167	151-167 (0405/-) 151-167 (DR4/8)	ND	ND
4	187-203	193-198 (0405/1301)	193-206 (0406/1301)	187-203 (0406/1301)
5	252-279	252-264 (0803/0801) 252-264 (DRB/-) 265-279 (0405/-)	ND	ND
6	283-314	283-293 (0405/-) 283-314 (0405/-) 283-314 (DRB/-)	ND	ND
7	345-362	348-353 (0803/0901) 352-358 (0803/0910)	345-358 (0803/0901) 349-362 (0603/0901)	ND

ND: not done.

DR antibody, but not by anti-DQ antibody, indicating their HLA-DR restriction (Fig. 4).

We demonstrated that all subjects with responsiveness to Cry j 2, including allergic and nonallergic donors, responded to very restricted regions of the allergen polypeptide regardless of HLA-DR haplotypes (Fig. 5). Furthermore, we did not find any disease-related T-cell epitopes. L1, L9, and V18, which were identified as positive fragments in nonallergic subjects, were pentamer/hexamers which appeared to be of minimum size in binding to the HLA-DR complex (18). These responses were confirmed by dose-response experiments. It is unlikely that the contaminating peptides in these fractions induced the positive responses because the final allergen concentrations in the *in vitro* assay were estimated to be extremely low. L1 (352-356) and V18 (348-353) are included in a 14-mer synthetic peptide (345-358) which gave positive responses in H. A. (Fig. 9). Similarly, the L9 (193-198) sequence was included in a 14-mer synthetic peptide (187-203) which induced a positive response in S. H. (Fig. 9). There were also cases in which allergic and nonallergic subjects responded to the same or overlapping peptide fragments (Table 2). This was determined with both enzyme-digested peptide fragments and chemically synthesized oligopeptides with over 98% purity. This phenomenon was previously reported in a study of the major birch pollen allergen Bet v 1, and in a study of the house-dust mite allergen Der p 1 (19, 20).

Thus, taking the results of the enzyme-digested fragments and the synthetic Cry j 2 peptides collectively, seven major T-cell epitopes were identified in

the Cry j 2 primary structure: 1-26, 70-84, 151-168, 187-203, 252-279, 283-314, and 345-362 (Table 3). Since M. H. showed a positive response to V27 (348-383), but not to V18 (348-353), there might be an additional sequence in the C-terminal region.

The proliferation of T cells is determined in two stages, i.e., the binding of T-cell epitopes to particular MHC class II molecules, and the generation of T-cell clones to bind the presented peptide. The "wobbling" in epitope specificity is permitted in both stages at the clonal T cell level. Thus, the response of the bulk population of T cells appears to reflect the mixed epitope specificities by the limited number of major T-cell epitopes.

Intact Cry j 2 required 0.1 μ M to reach the maximal response, whereas the synthetic peptide fragment (6-17-mer) required a 10-100-fold concentration (1-10 μ M). Furthermore, the magnitude of the response induced with the peptide fragment was relatively small. The variation of the triplicate responses was larger than that of intact Cry j 2 in PBL. These results imply not only a low frequency of anti-Cry j 2-specific T-cell clones, but also that there are other minor epitopes in addition to the major epitopes demonstrated in this study. These results suggest the sequential activation of Cry j 2-specific T-cell clones with different epitope specificities, a process referred to as epitope spreading (21). The utilization of APC expressing a higher level of MHC molecules (MHC gene-transfected cells or Epstein-Barr virus-transformed cells) may help to expand these minor T-cell populations *in vitro*, although their physiologic significance in the causation of allergy is uncertain.

A recent study showed that T-cell proliferation does not necessarily correlate with the magnitude of interleukin (IL)-4 gene expression that is directly involved in IgE antibody production (22). Since we surveyed T-cell proliferation in this study, there might be Cry j 2 peptides with low levels of activity for T-cell proliferation but showing immunodominance for IL-4 production. However, in a study on allergen Fel d 1 from *Felis domesticus*, peripheral T-cell tolerance to the entire molecule of Fel d 1 chain I was attained *in vivo* by subcutaneous injection of two 27-mer synthetic peptides which did not contain all the T-cell epitopes of Fel d 1 (23).

Altering the TCR ligand with a peptide analog on functional APC sends a signal to T-cell clones, resulting in anergy (5, 6). Prevention or mitigation of the allergic reaction might be attained by the inactivation of major epitope-recognizing T-cell populations in the early phase of the allergy. As Cry j 2 bears a limited number of immunodominant regions, this feature might lead to the molecular design of a Cry j 2 antagonist that could down-regulate the allergic reaction to Japanese cedar pollen and make practical immunotherapy feasible.

Acknowledgments

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